Transgenic Canola Plants Expressing cry1la5 Gene Are Resistant to (Agrotis Ipsilon) Using Sonication Assisted Agrobacterium Mediated Transformation (SAAT) Technique

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Abstract

The aim of this work was to transfer the *cry1la5* gene into the genome of two commercial canola cultivars using Sonication assisted *Agrobacterium* mediated transformation system and to test its toxicity against *A. ipsilon*. The integration and expression of the *cry1la5* gene into the plants genome was confirmed using PCR, Southern blot and Northern blot analyses. The insecticidal activity of transgenic plants against *A. ipsilon* was studied. The 2nd and 4th *A. ipsilon* larval instars were fed on leaves from five transgenic lines (L1, L2, L3, L4 and L5) for one week of T1 plants. Transgenic L5 was found to be more toxic to the 2nd larval instar causing 60% mortality on the 3rd day followed by 100% on the 4th day post feeding. While the other transgenic lines L2, L3, L4 caused 60, 20, 80% mortality respectively on the 6th day, whereas L1 did not show any observed toxicity to *A. ipsilon*. Digestive and detoxification enzyme activity were examined in the body of 2nd instar of *A. ipsilon*, fed on bt canola, showing significantly lower trypsin, protease, lipase, esterase and acetylcholinesterase activities and significantly increase Glutathione-S-transferase and Chitinase activities than non-bt fed larvae. Whilst the 4th larval instar fed on bt canola showed cannibalism as change in the behavior. The results of the study imply that transgenic canola lines obtained varying in toxicity against *A. ipsilon* which could be used to construct new canola cultivars resistant to *A. ipsilon*.

Introduction

Vegetable oils have long been an essential component of diet of humans. Today, the four main crops producing vegetable-oil are oil-palm, soybean, rapeseed and sunflower, respectively (USDA- FAS 2014). As an oil crop, rapeseed (*Brassica napus*) is widely used worldwide. It is also used as protein-rich livestock feed, as a source of biodiesel, lamp oil, soap, and plastics, an effective heavy metal hyper accumulator plant, as an excellent rotating crop for soil management, (Saeidnia and Gohari, 2012). One solution to improve the quality and quantity of canola products is the transfer of useful properties such as insect resistance and herbicide resistance through genetic transfer techniques (Tarinejad et al., 2013).

Because of transfer of low gene copy numbers and the transgene stability, *Agrobacterium tumefaciens* that contributes to gene technology is considered popular for genetic manipulation (Travella et al., 2005; Lee and Zhang, 2014). As the most effective protein insecticides used in agriculture, Bt delta-endotoxins coded by Cry genes of *Bacillus thuringiensis* are well known. These toxins are widely used as bacterial preparations, to control varied pest insects that are sprayed on field plots (Entwistle et al. 1993, Kuvshinov et al. 2001). Under field conditions these preparations are costly and degrade rapidly. Cry genes have been transformed into various plant species to grow insecticidal crops to solve these issues.

Selvapandiyan et al. (1998), cloned *Cry1la5* gene from an Indian isolate of *B. thuringiensis* and characterized it (EMBL accession number Y08920). *Cry1la5* provided full defense against the neonate stage larvae of *H. armigera*, When expressed in tobacco plants via nuclear transformation,. Moghaieb (2010), tested the transformation of rice plants with Cry1la5 against the third larval instars of *Chilo agamemnon* and concluded that it provides a good protection against the stem borer of rice.

The present study describes an effective protocol for the development of transgenic canola plants expressing the *cry1la5* gene resistant to black cutworm (*A. ipsilon*), which is one of the Egypt's major canola pests. The insecticidal activity produced by the transgenic plant against the larvae of *A. ipsilon* was examined.

Materials And Methods

Plant materials

Two canola genotypes, namely Serw3, Serw4 were kindly provided by Field Crop Institute, Agricultural Research Center, and Ministry of Agriculture – Egypt.

Canola seeds were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite, and rinsed in sterile distilled water. The sterilized seeds were germinated in flasks on 0.7% agar (w/v). The cultures were incubated at 25°C under a 16/8 h day/night photoperiod (1000-Lux) for subsequent uses in transformation (Moghaieb et al. 2006, 2014).

Bacterial strain

Professor V.S. Reddy (International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India) kindly given *Agrobacterium tumefaciens* strain LBA4404 containing the pBI-121 Cry1Ia5 plasmid (Selvapandiyan et al., 1998). The *Agrobacterium* was grown overnight in 30 ml of LB medium (Luria and Burrow, 1955) containing 50 µg/ml Kanamycin at 28°C (Fig. 1).

Sonication assisted *Agrobacterium* mediated transformation (SAAT)

The hypocotyl explants (0.5 cm in length) were isolated from 5-day-old canola seedlings (cultivars: Serw4 and Serw3) and were subjected to sonication for 5 seconds using Sonicor (model No./SC/152, N.Y) water bath, then immersed in bacterial suspension for 5 minute following Moghaieb et al. 2014 stated protocol. The explants were subsequently blotted on sterilized filter paper, placed onto a co-cultivation medium, which consisting of MS medium (Murashige and Skoog, 1962) supplemented with 1mg/l 2, 4-D and then incubated under dark conditions. The explants were transferred to supplemented shoot induction medium with 4.5 mg/l BA in addition to 50 mg/l kanamycin sulfate. The plates were parafilm- sealed and incubated at 26°C under a 16/8-h light/dark photoperiodic regime (1000-Lux).

Molecular conformation of the transformation events:

Complete DNA was extracted from both of *bt* transformed and non-transformed plants according to the method previously defined by Rogers and Bendich (1989) to confirm the stable incorporation of the *bt* gene into the resulting plantlet genome. Genomic DNA subjected to PCR analysis using 35S promoter specific primers, The sequence of forward and reverse primers 5'-AAA GGA AGG TGG CTC CTA CAA AT-3' and 5'-CCT AGT AAA GTA AAC CTC TCC-3', respectively. The reaction mixture (20µl) contained 10ng DNA, 200µM dNTPs, 1µM of each primer, 0.5 units of Taq polymerase (Thermo Fisher Scientific Inc.) and 10-X Taq polymerase buffer. Samples were heated to 94°C for 5 min, then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72°C. Via agarose gel electrophoresis, the PCR products were separated by and visualized with ethidium bromide. Plants were acclimatized and transferred in to the growth chamber to collect the T₁ seeds that showed positive result for 35S promoter.

Southern blotting analysis. Southern analysis was performed from genomic DNA of positive plants for the 35S promoter. In 2 % agarose gels, the digested DNA was separated, blotted to a nylon membrane following standard protocols (Sambrook *et al.* 1989). As a probe, the PBI-121-*Cry1Ia5* plasmid was used. Labeling of the probes, hybridization and detection were performed using the Biotin Chromogenic Detection kit #K0661, #K0662 according to instructions directed by the manufacturer (Thermo Fisher Scientific Inc.).

RNA dot blot and Northern blot analysis. Following the technique of Chrigwin et al. (1979), Total RNA was isolated from leaf samples of both the transgenic T₁ and control plants. Labeling of the probe, hybridization and detection was carried out using an RPN 3450 Gene Image kit in compliance with the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Insect bioassays on transgenic canola plants expressing Cry1Ia5

According to procedure of Parrott et al. (1994), detached leaf-insect bioassays have been carried out on T₁ plants. Petri dishes held single, detached two leaf discs were removed from the oldest non-senescing leaf on each plant, and were placed on moist filter paper in a Petri dish and the 2nd and the 4th larvae instars from black cutworms (*Agrotis ipsilon*) were placed on each plate of transgenic lines and non-transgenic control line with replicate plates. Under a 23-h photoperiod, the larvae were allowed to eat the plant material for 7 days and every day all the insects were subjected to weight. At the end of each 7-d experiment, insect survivorship data were obtained. After insects were collected and frozen at the end of the experiment, total protein determination and shift in enzyme activates in larval were measured.

Total protein determination in larvae (Protein assay)

Larvae were chilled on ice. The reaction was made according to method of Bradford (1976) and updated by Spector (1978). As standard protein, bovine serum albumin (BSA) was used. Spectrophotometer was used to read the reaction (absorbance) at 595

nm. Protein concentrations in mg/ml were determined from a standard curve of absorbance of known concentrations of bovine serum albumin.

Change in enzyme activities

According to Moghaieb et al. (2019) *Agrotis ipsilon* larvae have been used for hemolymph collection and enzymes extraction preparation. For the processing of hemolymph, Prolegs were cut and the hemolymph was dripped into microtubes. From each individual, approximately 25µl of hemolymph was obtained.

1. α-Esterase assay

As described in Asperen (1962) with minor modifications, esterase activity in the samples was determined spectrophotometrically. The mixture contained 1ml of 0.54 mM α- naphthylacetate in PB and 200µl of the sample. The concentration of α- naphthyl produced during the reaction was calculated spectrophotometrically at the wave length of 555 nm. 200µl of α-NA solution and 50µl of stain were included.

2. Acetyl cholinesterase (AChE)

AChE activity was measured according to Simpson et al. (1963). The reaction mixture contained 200µl enzyme solution, 500µl 0.067 M phosphate buffer (pH 7) and 500µl AchBr (3mM). The test tubes were incubated at 37°C for 30 min. 1ml of alkaline hydroxylamine chloride was applied to the test tubes. Then 500µl of HCl was added. The mixture allowed standing for 2 min. then 500µl of ferric chloride solution was added and mixed well and was assayed colorimetric at 515 nm. The decrease in AchBr due to hydrolysis by AchE was observed.

3. Glutathione S-transferase (GST)

Enzyme activity was estimated following the procedure of Habig et al. (1974) using 1-chloro-2, 4 dinitrobenzene (CDNB), and GSH as substrates. The absorbance level shift was noted at 340nm. Routine assays consisted substrate buffer of 1.82ml (100 mM potassium phosphate buffer pH 6.5), 40µl from 70 mM 1-chloro-2, 4 dinitrobenzene (CDNB) dissolved in 2 ml 95% ethanol to obtain final concentration (1.4mM); 40µl 70mM reduced glutathione as a cofactor in 2ml distal water to be 1.4mM .

4. Trypsin assay

As defined by Erlanger et al. (1961) the activity of trypsin was calculated using alpha-benzoylDL- arginine-p-nitroanilide (BapNA) 4mM N α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) in 7% Dimethylformamide (DMF) was diluted with 50mM Trizma hydrochloride (pH 8.5) then incubated with the sample for 30 min. at 37°C. At 410 nm, the rate of nitroaniline formation was calculated.

5. Chitinase activity

According to Ishaaya and Casida (1974), colloidal chitin was prepared. The reaction mixture with some modifications, according to Bade and Stinson (1981) consisted of 1ml phosphate buffer (0.2 M, pH 6.5), 200µl 0.5% colloidal chitin and 200µl enzyme solution. After 1.5 hour incubation at 37°C, enzyme activity was terminated by boiling test tube. Undigested chitin was sedimented at 8.000 rpm by centrifugation for 15 min. The supernatant was taken for determination of N- acetylglucoseamine produced as a result of chitin digestion by the sensitive method of Waterhouse *et al.* (1961).

6. Lipase determination

As defined by Tsujita et al. (1985), lipase assay was carried out. Thirty microlitre of gut extracts and 100µl of p-nitrophenyl butyrate (PNPB, 27 mM), as substrate were incorporated, mixed thoroughly and added at 37°C (Obtained by preliminary test). After 10 min, 100µl of distilled water were applied to each tube and absorbance was read at 405 nm. (Zibae and Fazeli-Dinan, 2012)

Statistical Analysis

In order to compare the protein content and enzyme expression levels between the control and the treated ones, biochemical data was subjected to a one- way analysis of variance (ANOVA) levels of statistical significance were determined at < 0.05. Observed

differences between the control and the treated ones were evaluated by independent t-test (SPSS version 9.0).

Results

The well proliferated explants derived from two canola cultivars (Serw3 and Serw4) were subjected to sonication for 5 seconds, in order to produce canola plant resistant to the black cutworm (*A. ipsilon*). According to the protocol defined by Moghaieb et al. (2014), high frequency of transgenic shoots production results. Before the co-cultivation with *A. tumefaciens* strain LBA4404, The explants were moved to MS medium containing 1mg/l 2,4-D for callus induction (Moghaieb et al., 2006). Followed by regenerating media containing 4.5 mg/l BA for two weeks during the selection culture; the explants were sub-cultivated into fresh medium containing 50 µg/ml kanamycin. Table (1) revealed the transformation percentages were 25 and 36% for serw3 and serw4 respectively.

Serw4 T0 plants that can live at 50 µg/ml kanamycin containing medium were subjected to PCR analysis using primers specific to the 35 S-promoter to confirm the stable incorporation of the T-DNA into the putative transgenic plantlet genomes because serw4 gives the highest transformation percentage. Figures (2) showed that a clear band at 250 bp was observed only in transgenic plants, but no such bands were seen in the non-transformed controls under similar conditions. In the presence of 50 mg/l kanamycin sulfate, the transformation efficiency of the two tested canola cultivars after selection was 25 and 36%, with a total of 30 and 90 transgenic plants derived from the cultivars Serw3 and Serw4, respectively. Using Southern analyses, the integration of the *Cry1la5* gene in the T₀ plant genomes was confirmed. As indicated in figure (2), the Southern analysis data suggest that single or two copy of the transgene found in most of the transgenics.

The expression of *Cry1la5* gene was investigated by northern blot analyses using the T1 plants to detect transcripts that hybridized directly to *Cry1la5* gene derived probe and accumulated in the transgenic T₁ plants which could not be detected in the non-transgenic plants (Fig. 3)

The Insect Bioassay to transgenic canola plants expressing the *Cry1la5* gene:

Selected northern positive T1 plants (showing normal morphology and growth) were assessed for to black cutworm (*A. ipsilon*) resistance and control plants were also included according to Kuvshinov et al. (2001). Transgenic plants were put on a moistened filter paper disc in a 90 mm-diameter Petri dish and infested with two larvae of black cutworm per Petri dish. Then Petri dishes were incubated at 26°C in the dark and, leaf cuttings from the same transgenic line were supplied twice a day for one week.

The insect weight increased from line 1–5 until the 5th day after feeding with transgenic material and then substantial decrease in their weight was observed compared to the control feeding insects (Fig. 4) which increased in weight with days of experiment.

The data obtained for the insecticide activity of five transgenic lines showed that the transgenic line 5 was more toxic to the 2nd larval instar of black cutworm (*Agrotis ipsilon*). On the 3rd day it resulted in 60% mortality followed by 100% on the 4th day. The other transgenic lines L2, L3, L4 triggered 60, 20, 80 percent mortality respectively on the 6th day and then the mortality rate for line 2 increased to 80% on day 7 the (Figs. 5 & 6), where no mortality was shown by the control plants. This suggests that transgenic canola plants have been toxic to *Agrotis ipsilon*, and may serve as genetic resource for canola resistance to insects.

Changes in the Protein content in the 2nd larval instar fed on transgenic canola

From the 2nd larval instar that had been fed on transgenic canola plants expressing the *Cry1la5* gene from five transgenic lines and from the control plants, total protein was extracted and analyzed. It should be noted that significantly lower protein levels have been observed in *Agrotis ipsilon* fed on *Bt* transgenic canola lines compared with the control canola ($P < 0.0001$) (Fig. 7). Compared with the other lines and the control, L5 displayed the greatest decrease in protein content which was associated with decreasing in the insect's weight.

Activities of the digestive enzymes in the 2nd larval instar fed on transgenic canola contain *Cry1la5* gene:

Trypsin:

In most insects, Serine proteinases, such as trypsin, chymotrypsin, are the key digestive alkaline endopeptidases and are essential both in the solubilizing and activating of Bt protoxins (Oppert, 1999; Terra and Ferreira (2005). The proteinases like trypsin derived from the larvae that fed on transgenic lines showed substantial decrease from that fed on control line ($P < 0.0001$) Fig. (8), and the decrease observed in L2 and L5 which was associated with the decrease in the protein content of both lines.

Protease:

As insecticidal agents, Proteases are logical candidates for use. Proteolytic enzymatic action can target and destroy essential proteins and tissues to an extent that mortality would result (Harrison and Bonning 2010). As shown in Figure (8), The protease derived from larvae fed on transgenic canola lines significantly decreased compared with that fed on the control line ($P < 0.0001$).

Lipase:

Lipids in insects are involved in many physiological functions like molting during larval and adult development (Kawooya and Law, 1988), reproduction (Majumder and Sengupta, 1979), energy supplement during starvation (Ziegler, 1991) and immunity. In the 2nd instar of *Agrotis ipsilon* fed on Bt transgenic canola lines, lipase activity decreased compared to fed on the control canola ($P = 0.0001$), and the reduction was more pronounced in insects fed on line 4 and line 5 as the weight of the insect decrease in those two lines (Fig. 8).

Activities of protection and detoxification enzymes in the 2nd larval instar fed on transgenic canola contain Cry1Ia5 gene:

Estrase:

In conferring or leading to insecticide resistance in insects, esterase enzymes play a significant role (Claudianos et al. 1999; Taskin and Kence, 2004). Enzyme synthesis reduction is due to the direct impact of toxicants on the synthesis and can be used as a marker for resistant individuals in populations of *B. tabaci* (Wool and Greenberg 1991, Kurappasamy et al., 2001).

The data presented in Figure (9) Suggest that the α - esterase concentration in the larvae fed transgenic leaves from line 1 and line 2 compared to the control was reduced. Compared to the control, while the insects fed on transgenic lines 3, 4 and 5 showed an increase in the enzyme activity. These results support our mortality's data or higher insecticidal activity that was found in transgenic lines 3, 4 and 5 (Fig. 9).

Acetylcholinesterase (AChE):

Acetylcholinesterase (AChE) is an essential enzyme that terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (ACh) in the nervous system of insects (Soreq and Seidman, 2001).

In the 2nd larval instar which fed on transgenic plants, the AChE activity reduced compared to the control ($P < 0.0001$). In larvae fed on line 4 and line 5, the reduction was more remarkable (Fig. 9). And this could clarify that the percentage of mortality on line 4 and 5 was higher than the other lines (Fig. 9).

Glutathione-S-transferase:

Current data suggest substantial increase in the GST extracted from larvae fed on bt canola relative to control canola ($P < 0.0001$), while line 1 decreased compared to the other lines (Fig. 11). Insect fed on transgenic line 5 displayed higher GST content followed by line 4 then line 3 and suggesting that these insects appear to increase GST concentration in their body for detoxify the Bt toxin from their body. These data is confirmed by the data provided in (Fig. 9) whereas transgenic line 3, 4 and 5 show higher mortality percentage.

Chitinase:

The activity of chitinase extracted from larvae fed on Bt expressing plants, increase extremely significant compared with fed on the control ($P < 0.0001$). in But line 1 and 3 displayed a decline in chitinase activity because of the insects in the two lines molt

earlier than all the lines and the wild type (Fig. 9).

The analysis of variance of different enzymes is shown in Table (2). The differences between insects that were supplied by transgenic material from different lines and the control plants were extremely significant for total protein contents, trypsin, protease, lipase, α -esterase, AchE, GST and chitinase activity.

Evaluation of Cry1la5 expressing canola plants against the 4th larval instar of black cut worm

The killing and consumption of all or part of a conspecific is called Cannibalism (Polis 1981, Elgar and Crespi 1992). Many density-dependent and density independent factors can influence.

Compared to that fed on the non transgenic plant material the 4th larval instar consumed much less transgenic leaves in the present study. The larvae started to fast and their behavior changed and began to cannibalize on the other larvae found in the same Petri dishes in the presence of canola leaves. In order to prevent the stress imposed by bt toxin, some larvae were forced to pupate, but the other insect did not pupate began to feed on the insect's pupa (Fig. 10).

In the body of the 4th instar larvae fed on transgenic plant leaves, there is an increase in the esterase activity measured. The data indicate that this enzyme activity was increased in the larvae fed on bt expressing plants compared to the control, which led to increased resistance to the bt toxin as esterase enzyme considered to be detoxifying enzyme and is essential in the resistance to insecticides and plant secondary substances (Klowden, 2007).

Discussion

Several plant transformation methods have been recorded for crop improvement program and can be applied to generate transgenic plant species, including agrobacterium and biolistic gun (John, 1997, Moghaieb 2010, 2014). In term of optimizing *in vitro* tissue culture and gene transformation, *Brassica napus* serves as suitable host for several new characteristic in is highly genotype based (Mashayekhi et al., 2008; Burbulis et al., 2010). Plants transformed by bt genes have varying degrees of defense against various insects. (Chen et al. 2007, Rahman et al. 2007)

The present study indicate that the expression of the *Cry1la5* gene was confirmed by Southern, RNA dot blot and northern blot analyses using T0 and T1 plants.

The mortality percentage of the five transgenic was checked on black cut worm and found that L5 was more toxic to larvae causing mortality % up to 100% on the 4th day followed by L2, L3, L4 causing 60, 20, 80% mortality on the 6th day as the copy number in Southern blot indicated, while the control showed 0 % mortality and these data agrees with findings of Cao et al. (2008) as four *Cry1C Brassica juncea* plants showed complete resistance to susceptible *Plutella xylostella* with 100% mortality. Also the finding agrees with Moghaieb (2010), when using the transgenic rice lines expressing *Cry1la5* gene against rice stem borer (*Chilo Agamemnon*) and recorded mortality rate of 100% four days post treatment, where as our findings disagree with Silva *et al.* (2016) as the Bt transgenic soybean had no impact on *S. cosmioides* as there is up to 80 % of larval survival.

The Changes in the Protein content in the 2nd larval instar of *A. ipsilon* fed on transgenic canola was lower than that fed on control plants and this decreases in haemolymph protein content could be caused by DNA and RNA synthesis inhibition, Mitlin et al. (1977) reported that there were also results consistent with Guo et al. (2011) in boll weevils treated with chitin synthesis inhibitors as there were significantly lower protein levels observed in *Spodoptra exigua* fed on *Bt* cotton for 1 h than those for 4, 6 and 24h.

The decrease in digestive enzymes like Trypsin was agreed with finding of Li et al. (2004) as the specific activity of trypsin-like proteinases in the soluble fraction of Bt-resistant *O. nubilalis* larvae was substantially lower than those of the susceptible larvae fed on diets containing doses of Bt *subsp. Kurstaki*. Guo et al., (2011) also found that the activity of trypsin in *S. exigua* after being fed on Bt cotton and non-Bt cotton for 24 h decreased significantly. The lipase activity was substantially lower in the experiment result of the use of bt expressing plants convenient with Zibae et al. (2010), as that *B. thuringiensis* and plant

extracts lowered the amount of lipase activity, also in accordance with Guo et al. (2011) who noticed decrease in lipase in *S. exigua* fed on Bt cotton for different times compared with those on control cotton for the same periods.

We conclude that the concentration of esterase enzyme has been decreased by analyzing the activities of defense and detoxification enzymes in the 2nd larval instar fed on transgenic Cry1Ia5 canola plants. Abuldahab et al. (2011), agree with our data finding that the activities of α – and β – esterases have decreased in larvae treated with *B. thuringiensis israelensis* and the enzymatic activities reduction were highly significant.

Acetylcholinesterase (AChE) demoralization results in excessive accumulation of ACh, leading to hyperactivity and eventually paralysis and death (Soreq and Seidman 2001). This explains the decrease in (AChE) in larvae fed on line 4 and line 5 which Guo et al. (2011) agreed with as the AChE activities were significantly lower in *S. exigua* fed on Bt cotton compared with those fed on non-Bt cotton for the same periods.

Glutathione-S-transferase (GST) enzymes play an important role in detoxification and protection against xenobiotic compounds including insecticides. Shuanyang *et al.*, (2001) found that after 24 hours of feeding the American white moth larvae with transgenic poplar, the activity of (GST) increased significantly and concluded that the detoxification enzyme mechanism in the midgut was inhibited and further toxic reaction of Bt protein could occur, this in line with our findings as there is substantial increase in the GST in insects fed on Bt plants.

Chitinases are enzymes that in many species are widely distributed and play roles in immunity and protection, digestion, pathogenicity and moulting of arthropods (Arakane and Muthukrishnan, 2010). Data showed that the activity of chitinase extracted from larvae fed on Bt expressing plants compared with fed on the control increase extremely significant and this agrees with Rashad et al. (2015) who worked with four insecticidal compounds on the 4th instar larvae of Pink bollworm and found that chitinase enzymes were increased in moderate percentages in both *Teflubenzuron* and *V. lecanii* compound although these disagree with the conclusion of Al-shannaf et al. (2012) that chitinase activity was increased in *H. armigera* as compared to the biopesticides, uses chlorfluazuron and pyriproxyfen as insect growth regulators.

The propensity to cannibalize, include many factors like, developmental asynchrony with host plants, sex or genetic relationships of the participants, food quantity or quality limitations, and abiotic environmental factors such as humidity and high temperatures. (Richardson et al. 2010).

In the study, the change in the behavior of 4th larval instar of black cut worm was studied to find that the insects' began to cannibalize on the other insects in the presence of the canola leaves confirming the finding of Richardson et al. (2010), and this finding need some study to understand either bt plants have negative effects on the behavior of the targeted insects or not. Our findings explains the rise in the esterase enzyme as it is known to be a detoxifying enzyme due to insect's resistance to insecticides as postulated by Klowden (2007).

Conclusion

The present study indicated that SAAT transformation technique has the potential for producing transgenic canola lines varying in toxicity against *A. ipsilon*. These transgenic materials can be used to construct new canola cultivars resistant to *A. ipsilon* to be used in IPM programs and to minimize the use of chemical pesticides.

Abbreviations

SAAT: Sonication assisted Agrobacterium mediated transformation

AChE: Acetylcholinesterase

GST: Glutathione-S-transferase

ANOVA: analysis of variance

PNPB: p-nitrophenyl butyrate

BApNA: N α -benzoyl-DL-arginine-p-nitroanilide

DMF: Dimethylformamide

CDNB: 1-chloro-2, 4 dinitrobenzene

BSA: bovine serum albumin

Declarations

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Competing of interests

On behave of all authors, the corresponding author states that there is no conflict of interest.

Availability of data and materials

All datasets are presented in the main manuscript.

Authors'contribution

Etr H. K. Khashaba: participated sufficiently in the work and manuscript writing.

Reda E.A. Moghaieb: experimental design, analysis, interpretation of data and manuscript writing.

Sawsan S. Youssef: participated in manuscript editing.

Sayed A. Morad: participated in the part of insect culture and bioassay.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1. The transformation frequencies of the two canola cultivars transformed with *Cry1la5* gene.

Cultivar	No. of calli	No. of regenerated shoots	No. of Kan.+ regenerated plants	Molecular conformation using PCR primer specific to 35S promoter	Transformation %
Serw-3	400	120	30	30	25
Serw-4	400	250	90	90	36

Table 2. Analysis of variance showing the differences in the enzyme activity between five transgenic canola lines expressing the *CryI1a5* gene against black cutworm (*A. ipsilon*).

S.V	d.f	Total protein MS	Trypsin MS	Protease MS	Lipase MS	α - estrases MS	AchE MS	GST MS	Chitinase MS	F 0.05
Replicates	2	0.1354 ^{ns}	2.793 ^{ns}	34.802 ^{ns}	944.22 ^{ns}	277.72 ^{ns}	3.921 ^{ns}	4.324 ^{ns}	208.39 ^{ns}	4.1
Treatment	5	10.327 ^{**}	33.413 ^{**}	1522.2 ^{**}	13258 ^{**}	24940 ^{**}	42.305 ^{**}	272.48 ^{**}	122593 ^{**}	3.32
Error	10	0.4714	1.237	25.329	746.69	823.26	1.029	2.434	1464.4	
Total	17									

Ns= non significant, ** = highly significant

Figures

Figure 1

Schematic representation of the pBI-121 Cry1Ia5 plasmid harboring the Cry1Ia5 gene under the 35SP and the NOS terminator used in canola transformation (Selvapandiyan et al., 1998).

Figure 2

Molecular confirmation of Cry1la5 gene integration into transgenic canola genome. A: represent PCR product. B: Southern blot analysis showing the integration of the Cry1la5 gene into canola genomes, 1: positive control, 2, 3: Transgenic L2, L3, 5, 6: Transgenic L4, L5.

Figure 3

Molecular confirmation of Cry1la5 gene integration into transgenic canola genome A: Acclimatized transgenic canola plants B: northern blot analysis showing the integration of the Cry1la5 gene into canola genomes. 1-3: represent transformed canola plants L2, L4, L5 and C: represent Negative control.

Figure 4

Insect's weight during the experiment time fed on transgenic plant material and control each plates contain two 2nd larvae instars.

Figure 5

Show the morphological change to the black cut worm. A. shows the body color of the control insect and (B) the insect fed with Cry1la5 transgenic material.

Figure 6

Mortality % in insects fed on transgenic lines during the experiment time.

Figure 7

Changes in protein content in the 2nd larval instar fed on transgenic lines and control one. W.t. = non transgenic, L1-L5= transgenic lines.

Figure 8

Change in trypsin, protease and lipase activity in the 2nd larval instar fed on transgenic lines and control one. W.t. = non transgenic, L1-L5= transgenic lines

Figure 9

Change in Alpha esterase, Acetylcholinesterase, Glutathione-S-transferase and Chitinase activity in the 2nd larval instar fed on transgenic lines and the control one. W.t. = non transgenic, L1-L5= transgenic lines.

Figure 10

Show the change in the behavior of black cut worm (cannibalism). A: the larvae eat the other larvae. B: the larvae cannibalize on the pupa. C: part from the insect and D: the larvae and pupa.